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14. ABSTRACT

The goal of this program is to investigate the influence of controlled mechanical stimulation on the behavior of progenitor cells in an effort to develop strategies to significantly enhance the rate and quality of fracture repair in long bone. Progress in the program has been excellent. The results to date demonstrate the application of load increases the callus volume, bone mineral density and biomechanical properties. More importantly, the data demonstrates a substantial independence on the time of load application. Load stimulation can positively influence fracture repair when applied at 10 or 24 days after fracture, while early application (during granulation tissue formation) may be detrimental to tissue regeneration. We also demonstrated that introduced progenator cells play an indirect role on the repair and identified a variety of factors that may be associated with repair cell recruitment. The results are beginning to support new strategies for enhancing fracture repair

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Table of Contents

Introduction

The goal of this program is to investigate the influence of controlled mechanical stimulation on the behavior of progenitor cells in an effort to develop strategies to significantly enhance the rate and quality of fracture repair in long bones. In support of these goals, we will test the global hypothesis that the migration, proliferation and differentiation of systemically or locally delivered MSCs is temporarily dependent on local mechanical conditions within the regenerate tissues.

Body

The progress of this research program is described below, as a function of the statements of work that were approved by the USAMRMC. The statement of work was proposed as follows:

1. Acquisition of transgenic GFP rats and establishment of a small colony for cell donation. This will be accomplished in the first eight months of the study.

2. Extraction, isolation and expansion of MSC from transgenic GFP rats to establish baseline of GFP signal in culture. This will occur during year 1.

3. Delivery of MSCs from GFP rats into wild type rats after treatment with F^{18}. This will be a dosing and cell viability study using microPET imaging and will be accomplished during year 1.

4. Fabrication of the required external fixation devices, associated pins and surgical guides will be performed during years 1 through 3.

5. Implementation of the first primary experiment: 108 rats with bilateral femoral 2mm defects and fixation will be entered into the study to evaluate the effect of load and systemic cell delivery on cell migration, using microPET scanning. Animals will be entered in year 1 through year 2.

6. The evaluation of the effect of delivery and mechanical stimulation on bone regeneration using histologic, micro-imaging and biomechanical assays will be performed in years 1 through 2.5.

7. 144 animals will be entered into the second primary experiment to evaluate the effect of local cell delivery and mechanical stimulation during years 2.5 through 3.5.

8. Complete analysis of the combined effects of local or systemic cell delivery with mechanical stimulation will be completed during years 3.5 through 4.

Progress during year three has been excellent. While the results of the first group of studies completed years 1 through 2 caused us to slightly alter our experimental methods and design, the overall objectives remain intact and the data has been very interesting. A summary of the results and accomplishments are described below, referenced to the original statements of work.

1. Acquisition of transgenic GFP rats and establishment of a small colony for cell donation. This will be accomplished in the first eight months of the study.

2. Extraction, isolation and expansion of MSC from transgenic GFP rats to establish baseline of GFP signal in culture. This will occur during year 1.

3. Delivery of MSCs from GFP rats into wild type rats after treatment with F^{18}. This will be a dosing and cell viability study using microPET imaging and will be accomplished during year 1.

4. Fabrication of the required external fixation devices, associated pins and surgical guides will be performed during years 1 through 3.

5. Implementation of the first primary experiment: 108 rats with bilateral femoral 2mm defects and fixation will be entered into the study to evaluate the effect of load and systemic cell delivery on cell migration, using microPET scanning. Animals will be entered in year 1 through year 2.

As described in the year 2 progress report, all of the studies associated with these tasks have been completed. It is important to remember that these studies demonstrated that the rate of systemic cell migration was not consistent with the use of MicroPET analysis and we altered our methods to utilize SPECT scanning with Indium ^{111} labeling of the MSCs. While we reported on some of the SPECT studies in the year 2 progress report, these studies were completed during this past year and will be summarized below. In addition, while the 2nd year progress report provided many details of the results from the first 108 animals, not all of the outcome measures were complete. During this past year, all of the assays and analyses were completed and the final results will be described below.

4. Fabrication of the required external fixation devices, associated pins and surgical guides will be performed during years 1 through 3.

5. Implementation of the first primary experiment: 108 rats with bilateral femoral 2mm defects and fixation will be entered into the study to evaluate the effect of load and systemic cell delivery on cell migration, using microPET scanning. Animals will be entered in year 1 through year 2.

6. The evaluation of the effect of delivery and mechanical stimulation on bone regeneration using histologic, micro-imaging and biomechanical assays will be performed in years 1 through 2.5.

All 108 animals completed the study as designated by the original experimental design (except that the imaging studies were performed on a separate group of animals). To review, the experimental design is summarized in Table 1. Note the specific variations in load initiation time (post-surgery) and sacrifice time.

TABLE 1

Group (total # animals)	Surgery Day	Loading initiation and cell delivery	Euthanasia Day 10 (# animals)	Euthanasia Day 24 (# animals)	Euthanasia Day 48 (# animals)
A (36)	0	0	(12)	(12)	(12)
B (36)	0	3	(12)	(12)	(12)
C (24)	0	10	-	(12)	(12)
D (12)	0	24	-	-	(12)
108 total animals					

As described in the 2nd year progress report, the loading was performed using a computer controlled axial loading system and included a sinusoidal waveform at 0.5 Hz. for 17 minutes for a total of 510 cycles at 8% global strain in tension and compression.

All animals had cell injections systemically by tail vein injection. During the past year, we finished all of the analyses including; MicroCT, biomechanical testing and histologic evaluation on all 108 animals. Importantly, since our early analysis suggested a significant effect of load on progenitor cell migration and homing, **we added a new, modest experiment** to begin a search for specific biologic factors that might be responsible for the "homing" response. This experiment included the following:

12 rats were divided into 4 groups:

Group 1: bilateral fixators/osteotomies, loaded on days 3-7, euthanized day 7.
Group 2: unilateral fixator/osteotomy, no load, euthanized on day 7.
Group 3: bilateral fixators/osteotomies, loaded on days 24-28, euthanized day 28.
Group 4: unilateral fixator/osteotomy, no load, euthanized on day 28.

Immediately after each animal was euthanized, the callus tissue was removed under sterile conditions. The legs of each animal were shaved, the femora were

exposed and harvested, and the fracture calluses were removed from the osteotomy using a scalpel blade. After each callus was removed, it was immediately placed in an RNase-free microfuge tube and snap frozen in liquid nitrogen. The harvested tissues were then stored at -80°C until they were processed for RNA extraction. Using standard procedures, the RNA was extracted and then processed for RT-PCR Array analysis. The concentration of RNA for each sample was determined by spectrometer. All of the samples except one, which had a lower concentration, were diluted to a concentration of 126 ng/µl. Samples were examined on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, California) to determine the integrity of the RNA. Two, 5µl amounts of each sample were alloquated for analysis, and all experiments were performed in duplicate. An RT2 ProfilerTM PCR Array System (all components from SABiosciences, Frederick, MD) was used for PCR analysis. The RNA in the 5µl samples was converted to cDNA using the RT2 First Strand Kit, and the experimental cocktail for RT-PCR was created using the RT2 qPCR master mix. A 384-well, custom PCR array was used to determine the expression of selected genes. Each array contained four sets of a panel of 84 genes of interest (See Table 1 and Table 2), five housekeeping genes, and three RNA and PCR quality controls. The genes of interest were selected through a literature search and chosen due to evidence that they are involved in the migration of mesenchymal stem cells (1-27). The specific genes selected are illustrated in Tables 2 and 3.

Table 2 and 3: Gene candidates selected for PCR array

Table 2

Symbol	Description
Cxcl12	Chemokine (C-X-C motif) ligand 12
Il1a	Interleukin 1 alpha
Il1b	Interleukin 1 beta
Il6	Interleukin 6
Tnf	Tumor necrosis factor
Tgfb1	Transforming growth factor, beta 1
Tgfb2	Transforming growth factor, beta 2
Tgfb3	Transforming growth factor, beta 3
Pdgfa	Platelet derived growth factor, alpha
Pdgfb	Platelet derived growth factor, beta
Bmp1	Bone morphogenetic protein 1
Bmp2	Bone morphogenetic protein 2
Bmp3	Bone morphogenetic protein 3
Bmp4	Bone morphogenetic protein 4
Bmp5_predicted	Bone morphogenetic protein 5 (predicted)
Bmp6	Bone morphogenetic protein 6
Bmp7	Bone morphogenetic protein 7
Cxcl1	Chemokine (C-X-C motif) ligand 1
Gdf5	Growth differentiation factor 5
Gdf8	Growth differentiation factor 8
Fn1	Fibronectin 1
Vtn	Vitronectin
Col1a1	Procollagen, type 1, alpha 1
Angpt1	Angiopoietin 1
Angpt2	Angiopoietin 2
Vegfa	Vascular endothelial growth factor A
Vegfb	Vascular endothelial growth factor B
Vegfc	Vascular endothelial growth factor C
Igf1	Insulin-like growth factor 1
Cd44	CD44 antigen

Symbol	Description
Ihh	Indian hedgehog homolog, (Drosophila)
Bglap2	Bone gamma-carboxyglutamate protein 2
Igf2	Insulin-like growth factor 2
Fgf1	Fibroblast growth factor 1
Fgf2	Fibroblast growth factor 2
Fgf3	Fibroblast growth factor 3
Fgf4	Fibroblast growth factor 4
Fgf5	Fibroblast growth factor 5
Mmp2	Matrix metallopeptidase 2
Mmp7	Matrix metallopeptidase 7
Mmp8	Matrix metallopeptidase 8
Cx3cl1	Chemokine (C-X3-C motif) ligand 1
Cxcl16	Similar to chemokine (C-X-C motif) ligand 16
Mip1	Myocardial ischemic preconditioning 1
Egf	Epidermal growth factor
Hbegf	Heparin-binding EGF-like growth factor
Tgfa	Transforming growth factor alpha
Hgf	Hepatocyte growth factor
F2	Coagulation factor II
Ccl2	Chemokine (C-C motif) ligand 2
Ccl5	Chemokine (C-C motif) ligand 5
Ccl22	Chemokine (C-C motif) ligand 22
Lif	Leukemia inhibitory factor
Ntf3	Neurotrophin 3
Pgf	Placental growth factor
Cxcl10	Chemokine (C-X-C motif) ligand 10
Csf2	Colony stimulating factor 2 (gran-macrophage)
Csf3	Colony stimulating factor 3 (granulocyte)
Ibsp	Integrin binding bone sialoprotein
Flt1	FMS-like tyrosine kinase 1

Table 3

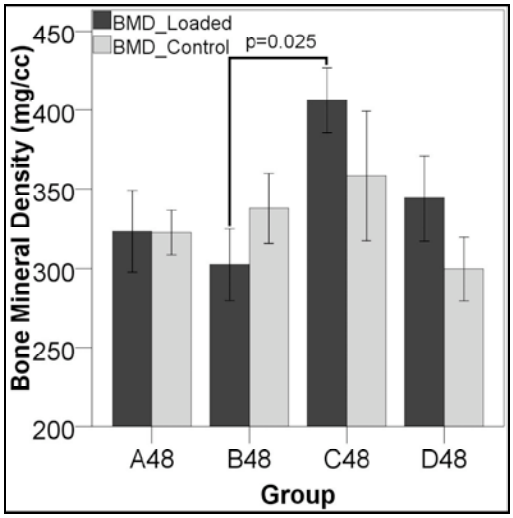
Symbol	Description
Cx3cr1	Chemokine (C-X3-C) receptor 1
Bmpr1a	Bone morphogenetic protein receptor, type 1A
Bmpr1b	Bone morphogenetic protein receptor, type 1B (mapped)
Bmpr2	Bone morphogenic protein receptor, type 2
Igf1r	Insulin-like growth factor 1 receptor
Igf2r	Insulin-like growth factor 2 receptor
Pdgfra	Platelet derived growth factor receptor, alpha polypeptide
Pdgfrb	Platelet derived growth factor receptor, beta polypeptide
Kdr	Kinase insert domain protein receptor
Ccr1	Chemokine (C-C motif) receptor 1
Ccr2	Chemokine (C-C motif) receptor 2
Ccr4	Chemokine (C-C motif) receptor 4
Ccr7	Chemokine (C-C motif) receptor 7
Il8rb	Interleukin 8 receptor, beta
Cxcr3	Chemokine (C-X-C motif) receptor 3
Cxcr4	Chemokine (C-X-C motif) receptor 4
Cxcr6	Chemokine (C-X-C motif) receptor 6
Fgfr1	Fibroblast growth factor receptor 1
Fgfr2	Fibroblast growth factor receptor 2
Fgfr3	Fibroblast growth factor receptor 3

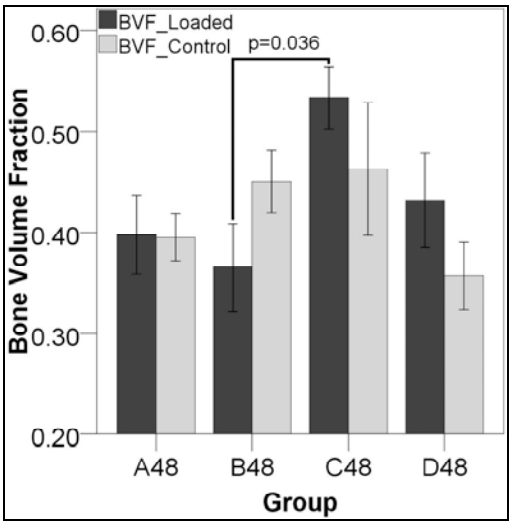
Results

A. Results for primary experiment in Phase I studies (MicroCT, biomechanical tests)

The results of the first phase analysis were dominantly described in the progress report for year 2. Since the entire cohort has now been completely analyzed, we will only summarize the major findings. In general there were no substantive changes to the results from those described in year 2.

The ratio of the stimulated to the control limbs shows decreases in all measures of mineralization for the groups displaced three days post-operatively. This decrease was as high as 19% in the BVF for animals euthanized at day 48. (Figures 1 and 2) All other groups showed an increase in mineralization in the stimulated fractures when compared to the unloaded controls. For rats that were sacrificed ten days after surgery (groups A10 and B10), there is a significant difference in the interaction of treatment and the timing of displacement. In those animals, displacement decreased both the callus volume and BMC in fractures stimulated starting on day three (group B10), while stimulation increased the same measurements in fractures stimulated immediately after surgery (group A10). The decrease between the loaded and control limbs within group B10 is significant for the callus volume, and the BMC within group A10 trends toward an increase ($p=0.074$).





Figures 1 and 2: BMD and BVF are significantly higher in Group C (day ten displacement) versus Group B (day three displacement) 48 days after surgery. In the later stages of healing, the mineral content is higher on the displaced side in the animals loaded ten days post-operatively than those at other stimulation time points. This reached a significant increase over the defects displaced three days post-op, since the mineral levels in those gaps were slightly depressed compared to the other groups.

When fractures are analyzed 24 days post-operatively, there is a strong trend for differences across all of the loaded limbs in BMC (p=0.055) and BMD (p=0.059). Looking for differences between individual groups reveals that there is less mineralized tissue in the displaced fracture gaps of the animals stimulated on day three versus the animals stimulated on either day zero or day ten. This difference reached significance for BMD between groups B (stimulation day 3) and C (stimulation day 10) (Figure 1). At day 48, there is a significant difference in the BMD (p=0.045) and BVF (p=0.049) across all the stimulated limbs (Figure 3). Individually, there is a difference between the group displaced three days post surgery (group B48) and the group displaced ten days post surgery (group C48), with the loaded defects from C48 having more mineral than those in B48. Along with the increased mineral content in the fractures stimulated ten days post-op, there is a significant decrease in cartilage in the healing defect for that group. The loaded limbs in group C48 have significantly less cartilage than their contralateral controls (p=0.045), while the loaded limbs in group A48 have more cartilage than there contralateral controls (p=0.037). There is also a difference in cartilage area between limbs stimulated at day ten and limbs that were stimulated at day zero (p=0.031) or day three (p=0.015). Stimulation starting at day three (group B10) induced more cartilage formation by day ten than did stimulation immediately after surgery (group A10). It also shows that the control limbs in the B10 group have a larger cartilage area than the controls from group A10 suggesting a possible systemic effect.

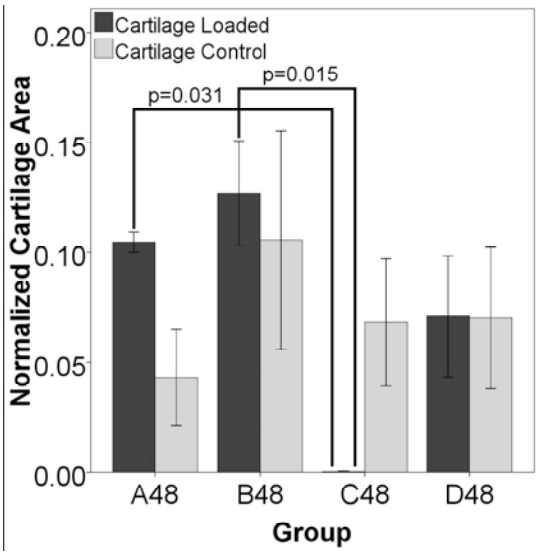


Figure 3: After 48 days, there is cartilage remaining in all groups, both stimulated and control, except for the gaps stimulated ten days after surgery (group C48). The differences were significant between the displaced limbs of A48 and B48 in comparison to C48. There is also a significant difference between the displaced and contralateral control in group C48 (p=0.045), with the displaced side containing significantly less cartilage than the control. There is also more cartilage in the displaced side in group A48 when compared to the contralateral control (p=0.037).

In animals euthanized on day 48, there is a significantly larger percentage of bone in the fracture gaps as measured by histology on both the loaded and control sides for the animals loaded starting on day ten (group C48) than for any other group.

B. Tracking of Progenitor Cells

As noted in earlier progress reports, we used SPECT imaging of Indium ^{111} labeled cells to track whether the progenitor cells would preferentially migrate to sites of repair and in particular to sites of mechanical stimulation.

A detectable number of MSCs are delivered to both femora after a systemic injection of cells as measured by planar gamma imaging. A high number of cells also remain in the visceral organs, especially the lungs, spleen, and liver. Over the course of the three days, the cells began to migrate towards the lower extremities. This pattern was not seen when indium^{111} was injected without first being incubated with MSCs. In the femora, immediately after injection (displacement day one) there is a strong trend (p=0.0586) toward more activity in the stimulated femora in comparison to the controls regardless of when displacement was initiated (Figure 4). On day one, there is significantly more activity in the stimulated limbs of the rats displaced on day three (group B) and a trend for more activity in the rats stimulated on day 24 (group D). On the second day of loading, the timing of displacement administration with respect to the systemic injection of cells had a significant bearing on the migration of the MSCs, regardless of side (p=0.0079 (Figure 5). The group in which displacement did not start until three days after injection (group E) had significantly less activity on both the stimulated and control sides as compared to groups B (displacement day three) and D (displacement day ten) and a trend toward less activity when compared to group A (displacement day zero). By the third day of scanning,

there were no differences within or between groups.

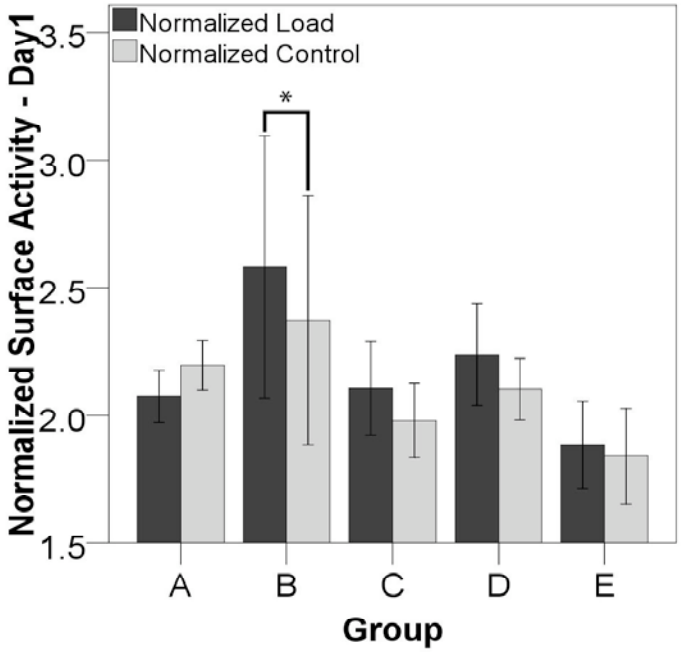


Figure 4: The normalized surface activity of the femora after the first application of axial displacement. The displaced limbs in the rats stimulated starting three days after surgery (group B) showed an increase in radioactivity when compared to the unloaded controls (*p<0.05). There was also a trend for an increase in the displaced limbs of the rats stimulated 24 days after surgery (group D) (p=0.0818).

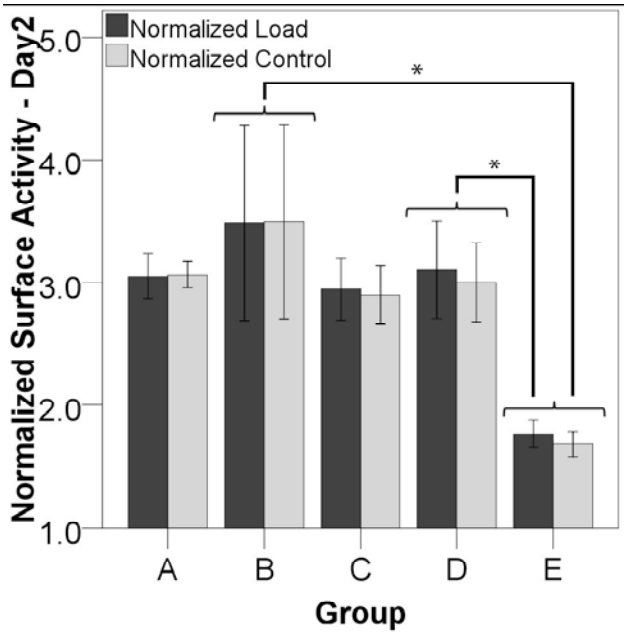


Figure 5: The group that was injected with MSCs after three bouts of displacement (group E) shows less activity in both limbs than in any other group. There was a significant difference for both the stimulated and control values across all groups. The effect of load initiation was also significant for groups B and D when compared to group E (there was a trend between groups A and E). This indicates that a smaller proportion of injected cells are available to both femora when the delivery of cells is delayed until axial displacement has already begun. (*p<0.05).

C. Immunohistochemistry analysis of GFP donor cell

In an effort to track the fate of the donor cells, we performed immunohistochemistry of the introduced cells. Since the cells were extracted

from the GFP transgenic rats, their fate or the fate of their daughter cells could be tracked.

IHC shows that even though there is a small number of exogenous MSCs present throughout the healing process, the largest population of cells does not appear until 48 days after surgery. At that time, MSCs are detected in large populations throughout the marrow in the medullary canal (Figure 6) and the marrow spaces within the periosteal callus (Figure 7). This is true for all of the groups euthanized on day 48 except for the group stimulated ten days post-op (group C). It also appears that stimulation slightly increases the number of cells in the fractured limb, as the scores for the stimulated limbs were slightly higher than the control limbs at most time points. These results likely suggest that the role taken by the cells is dominantly to express factors that help to condition the wound site as opposed to differentiating into matrix producing cells.

D. PCR array results

In animals that underwent axial displacement starting three days after surgery and then were euthanized on day seven, IGF-2, IGF-2 receptor, and Col1a1 were up-regulated in the loaded fracture gap versus the contralateral control gap. HGF and angiopoietin-1 were down-regulated in the loaded callus tissue when compared to the contralateral control. For animals that were stimulated starting on day 24 and euthanized on day 28, CXCL-10 (IP-10), BMP-6, Bglap-2 (BGPR), EGF, and Ihh were all up-regulated in the displaced fracture tissue versus the contralateral control. Several genes were also down-regulated in the stimulated callus in comparison to the control including IL-8 receptor beta, MMP-8, CX3CR-1, IL-6, and CSF-3. To test for effects that the displaced gap may be having on the distant, control fracture, the control fracture tissue from animals that had bilateral osteotomies was compared to the fracture gaps from animals that only had one, unstimulated osteotomy. HGF, CCL22 (MDC), and TNF-a were up-regulated in tissue from control fractures in bilateral rats stimulated starting day three in comparison to the fractures in the unilateral model. IBSP, GDF-5, Col1a1, Ihh, MMP-2, and Fn-1 were all downregulated in those animals. For animals euthanized on day 28, FGF-4 and CX3CR-1 were up-regulated in control fractures from the bilateral rats versus the fracture tissue from the rats with only one osteotomy. Ihh, BMP-6, CXCL-1, IBSP, FGF-3, CXCL-10 (IP-10), GDF-5, and CSF-3 were all down-regulated in the same comparison.

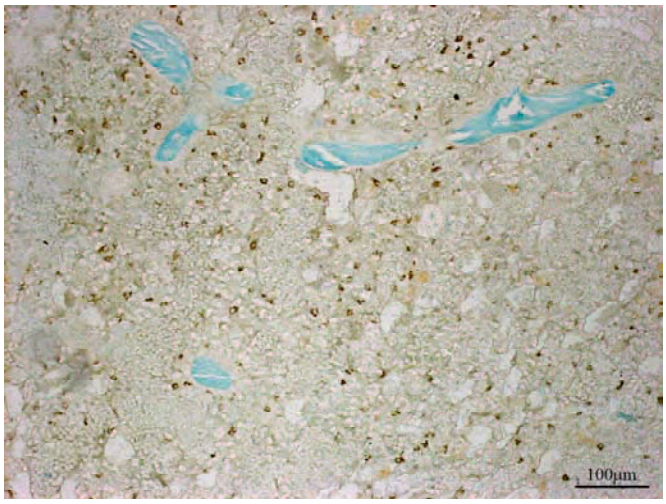


Figure 6: GFP positive cells in the marrow 48 days after surgery (brown stained cells), At all of the time points there was evidence of some GFP positive staining, but it was not until day 48 that there were large populations of GFP positive cells in the marrow spaces. Cells were also present in other locations (cortices and pin sites), but the most consistent location for the MSC populations was in the medullary marrow and the marrow within the periosteal callus.

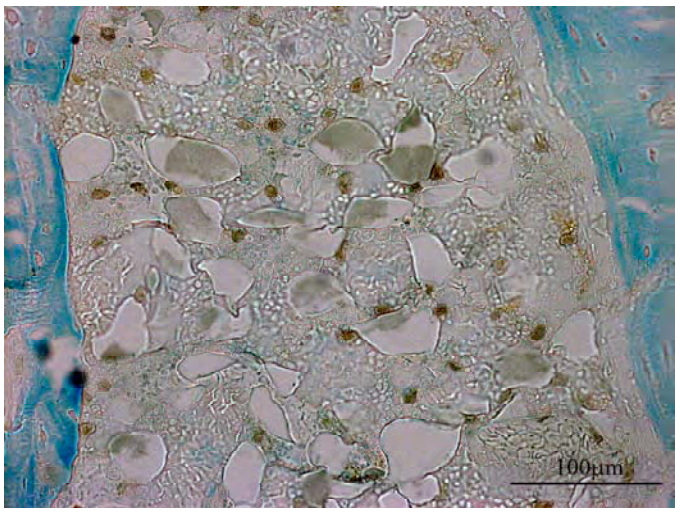


Figure 7: GFP positive cells in the periosteal callus (brown stained). The injected MSCs populated any area that consisted of marrow spaces. The areas in between the original cortex and the hard callus shell that formed from a periosteal response consist of marrow tissue. The injected MSCs were often found in these areas. The above image is of cells in the periosteal callus.

Discussion

The observation that the application of displacement on the animals soon after surgery decreased mineralization and mechanical properties in relation to the animals that had displacement starting on day ten suggests that axial mechanical stimulation may not be beneficial when it is started during the initial response to fracture. Vascular supply is an important factor in determining the success of healing, and it has been suggested that it may be necessary to allow neovascularization to progress at the site of repair before mechanical load is applied (28, 29). If motion is allowed too soon at the fracture site, the capillaries needed to support osseous tissues are constantly ruptured, and fibrocartilage formation is promoted since it requires less vascularization (30). Therefore, it may be beneficial to the overall healing outcome to delay initiation of loading until new vessels have had a chance to form (31).

The results also suggest that it may be beneficial to start fracture stimulation after the inflammatory stage, when some soft tissues have had a chance to form. After the initial inflammatory response, cells that may be responsive to load, such as chondrocytes (32,33), have an opportunity to populate the fracture site. A beneficial response to chondrocyte loading in fracture healing has been shown. Scaffolds seeded with chondrocytes that were implanted in the femora of rabbits and then were compressively loaded had a higher bone volume fraction than the unloaded controls (34), showing that the application of a stimulus to a chondrocyte population may encourage bone formation at the site of repair.

A controlled, axial stimulation has a definitive effect on fracture healing, and the timing of the application of the displacement differentially effects callus morphology and mechanical properties. Stimulation early in the repair process was not beneficial to fracture healing, but when the displacement was applied starting ten days after injury it increased mineralization, accelerated callus remodeling, and increased torsional mechanical properties in comparison to other groups. The beneficial effect was seen on both the experimental and the contralateral control defects, indicating that there may be a systemic effect from the applied stimulus.

Despite the fact that the cell number initially prevents the cells from moving freely through the circulation, some of the cells do find their way to the injured limbs. They do not localize to the injury directly, but instead the activity seems to encompass the entire femur. This may be due to the fact that the surgical procedure involves the placement of four bicortical pins to stabilize the fracture. These pins may act as additional sites of injury that encourage the cells to populate the region around the entire femur as opposed to localizing to the osteotomy. The planar gamma images also show a transient difference in the radioactivity detected between different groups. On the first day of axial displacement, the stimulation increases the activity in the loaded limbs versus the

unstimulated controls. That difference disappears by day two, when no differences between the two sides are observed. On day two though, there is a difference between all of the groups that underwent axial displacement immediately after cell injection and the animals in which cell injection was delayed until the fourth day of stimulation (group E). This may suggest a transient systemic effect of load timing in relation to when the cells are delivered, as activity on both sides of the late delivered cell group showed a decrease. This effect could be due to many factors. The extra sessions of axial displacement could have triggered the systemic release of antimigratory factors (or hindered the expression of migratory factors). Damage that could be induced by displacement could have accumulated over the course of the first three days and caused an adverse response. All of the radioactivity differences appear to be transient though, as there were no differences between any group by day three. The inability to detect differences by that time point may also be due to a decline in sensitivity, as the half-life of indium^{111} is about three days, which would reduce the overall signal that can be detected.

The sections stained for GFP through IHC confirmed that cells are able to migrate to the femora. It also showed that the injected MSCs were, in most cases, able to establish a population in the marrow by day 48. The exogenous MSCs were found in other locations other than marrow compartments, but it seems that the most consistent populations were found in the marrow. This is probably because the marrow and the cambium layer of the periosteum are considered primary stem cell niches (35) and so it is natural for the cells to engraft there as opposed to other locations. This is consistent with other studies that have found exogenous MSCs in the bone marrow for long periods after a systemic injection (36), and do not appear in large numbers in the first few weeks after delivery (37). Most of these other studies though involve pretransplant conditioning like irradiation of the host animal (38), while few have been able to show long-term engraftment after bone marrow transplants without conditioning.

Phase II studies

The phase II studies were designed to evaluate the influence of mechanical stimulation and local implantation of progenitor cells. The task associated with initiating Phase II was as follows:

7. 144 animals will be entered into the second primary experiment to evaluate the effect of local cell delivery and mechanical stimulation during years 2.5 through 3.5.

In order to perform the Phase II studies, we needed to develop the delivery matrices for the cells. As described in the original proposal, the matrix utilized was demineralized bone matrix (DBM). During the past year we developed the protocols for producing the DBM as well as tested the cell viability in the delivery

scaffold. In addition, we initiated the animal studies as proposed in task 7. The specific accomplishments are described below.

a. Protocols for producing DBM from Sprague Dawley rats was developed and tested.
b. A preliminary study was performed, in vitro, to ensure that the GFP MSCs would remain viable in the DBX. The results demonstrated that the cells were viable and the delivery technique was valid.
c. We developed and validated a technique for reproducibly creating constructs consisting of DBM and 1,000,000 cells in a 2mm. X 3mm. cylindrical geometry that could be delivered into the femoral defects.
d. We mass produced sufficient DBM scaffolds to complete the entire Phase II study and had them sterilized by gamma irradiation. All were maintained in sterile containers, ready for mixing with the cell constructs prior to surgical insertion.
e. **Most importantly, we have now entered approximately 80 animals into the Phase II studies**. Nearly all of the long-term animals have already been entered into the study as well as a number of animals across all the treatment groups.

In summary, we are on target for our Phase II studies. We expect to complete all of the surgical procedures within the next 2 months. In addition, the analysis of animals euthanized is occurring in parallel. This process will enable us to maintain the expected timetable.

Key Research Accomplishments

1. Completion of Phase I studies using 108 animals for MicroCT, biomechanical, histologic, nuclear imaging and immunohistochemical analysis of the temporal influence of load on fracture repair and enhancement with introduced progenitor cells. The results demonstrate:

    a. The timing of the application of a mechanical stimulus can play a role in the progression of healing in a fractured bone.
    b. An axially applied displacement had a beneficial effect on mineralization and the progression of remodeling in animals that were stimulated after ten days.
    c. Loading on day three after fracture was shown to be deleterious to the repair process.
    d. Mesenchymal stem cells, injected into these animals, took residence in the marrow spaces of all the animals after 48 days, with the exception of the animals stimulated starting on day ten.
    e. The data also suggest that exogenous cells (when given at certain times) may not be beneficial for fracture healing, as they may compete with endogenous cells or regulatory processes.

f. PCR array applied stimulus demonstrated substantial changes in the expression of several important factors, both locally and systemically.

2. Based on the findings of Phase I studies and analysis, it is clear that mechanical loading and the introduction of progenitor cells can have a substantial effect on fracture repair. However, the timing is critical and needs to be considered when developing novel treatment strategies.

3. Phase II studies are well underway and we have successfully implemented the use of a DBM construct for delivery of progenitor cells locally.

Reportable Outcomes

Aaron S. Weaver. The Effect of Mechanical Stimulation on Bone Fracture Healing: Changes in Callus Morphology and Mesenchymal Stem Cell Homing. Doctoral Dissertation. University of Michigan September 2008.

Weaver, Aaron S.; Alford, Andrea I.; Hankenson, Kurt D.; Su, Yu-Ping; Begun, Dana L.; Kreider, Jaclynn M.; Ablowitz, Stephanie A.; Kilbourn, Michael R.; Goldstein, Steven A. "Influence of controlled mechanical stimulation and donor mesenchymal progenitor cells on fracture healing." Transactions of the ORS 54th Annual Meeting, San Francisco, CA., volume 33, paper # 396, 2008

Weaver AS, Su YP, Begun DL, Miller JD, Alford AI, Goldstein SA. "The Effects of Axial Displacement on Fracture Callus Morphology and MSC Homing on the Timing of Application." To be submitted to the Journal of Orthopaedic Surgery.

In addition to these results, the data helped to attract additional funding for studies focused on the mechanisms of cell mechanoresponsiveness from the National Institutes of Health:

National Institutes of Health (R01AR51504), "Mechanical Factors and Cellular Responsiveness in Fracture Repair," (S. Goldstein, Principal Investigator), $1,125,000 TDC, 1/1/07-11/30/11.

Conclusions

As summarized in the body of the report, our studies revealed that the application of mechanical load can influence the character and rate of fracture repair. This influence, however, is dependent on timing of load application. In fact, loads applied at day three were shown to be detrimental to the repair process. This is likely due to the disruptive effect on early neovasularization and granulation

tissue formation. In contrast, loads applied after 10 days had a positive effect. Furthermore, the introduction of systemic progenitor cells resulted in mixed results. While the cells found their way, preferentially, to the repair site and when stimulated, their influence was not always positive. We hypothesize that this was due to a competition with endogenous cells or factors. Furthermore, immunohistochemistry demonstrated that the cells most often located to the marrow and likely contributed through the production of soluble factors and not by differentiating to matrix producing cells. Taken together, these results suggest that the use of mechanical stimulation and potentially systemically introduced cells, the fate of fracture repair can be substantially altered. With additional information, these results will likely lead to new strategies for enhancing repair as well as strategies to prevent a delay in the repair process.

Future studies and timetable

The program is on time and the proposed goals for year 3 have been met. The next studies follow the original research plan and involve the completion of Phase II studies using local implantation of cells with loading. These studies are already well underway and on target.

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